Crop Protection 112 (2018) 159–167

Contents lists available at ScienceDirect

Crop Protection

journal homepage: www.elsevier.com/locate/cropro

Developed and validated inoculation and disease assessment methods for Goss's bacterial wilt and leaf blight disease of corn

Atta Soliman[a,b], Robert H. Gulden[a], James T. Tambong[c], Pratisara Bajracharya[e], Lorne R. Adam[a], Renlin Xu[c], Morgan Cott[d], Fouad Daayf[a,*]

[a] Department of Plant Science, Faculty of Agricultural and Food Sciences, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada
[b] Department of Genetics, Faculty of Agriculture, University of Tanta, Egypt
[c] Agriculture and Agri-Food Canada, Ottawa, Ontario, K1A 0C6, Canada
[d] Manitoba Corn Growers Association, Carman, Manitoba, Canada
[e] Manitoba Agriculture, Manitoba, Canada

ARTICLE INFO

Keywords:
Corn
Clavibacter
Goss's wilt
Inoculation methods
Disease assessment

ABSTRACT

One of the drawbacks of conducting research on Goss's bacterial wilt and leaf blight disease, caused by the bacterium *Clavibacter nebraskensis* (Cn) on corn (*Zea mays* L.), is the lack of standardized and validated inoculation and disease assessment methods. Here we report foliar and root inoculation techniques with a standardized scale for reliable assessment of symptoms to allow for disease assessment of the Goss's wilt disease on corn. The leaf inoculation method allowed for the application of equal amounts of inoculum, which ensured consistency in measurements and prevented inoculum run off from the inoculation site. The reliability of a leaf inoculation method based on either lesion length or disease severity, and root inoculation based on disease severity was validated statistically using two Cn isolates, Cmn14-5-1 and DOAB232, which possess high and low levels of aggressiveness, respectively. The leaf disease-severity and the lesion length methods for disease assessment clearly provided the most precise estimation of the level of aggressiveness of Cn isolates among the tested years (2015, 2016, and 2017 growing seasons). In addition, the disease severity based on the root inoculation method was validated, but the high variation among experimental runs within each year suggested that it might be the least reliable method for assessment of the disease severity. Our results indicated no significant trend in overall isolate aggressiveness over the years using the leaf inoculation method based on either lesion length or leaf disease-severity.

1. Introduction

Different species of the phyto-bacterial pathogen *Clavibacter* cause high yield losses in economic crops (Eichenlaub and Gartemann, 2011). Among them, *Clavibacter nebraskensis* (Cn), the causal agent of Goss's wilt and leaf blight disease of corn (Vidaver and Mandel, 1974; Schuster, 1975), was first isolated in 1969 and designated as a new species, under the name of *Corynebacterium nebraskense* (Vidaver and Mandel, 1974), which was changed to *Clavibacter michiganensis* subsp. *nebraskensis* and recently to *Clavibacter nebraskensis* (Cn) (Li et al., 2018). The pathogen overwinters in corn stubble and alternative plant hosts such as barnyardgrass (*Echinochloa crus-galli*) (Wysong et al., 1981), foxtail (*Setaria spp.*), shattercane (*Sorghum bicolor*) (Schuster, 1975; Langemeier et al., 2014), annual ryegrass, johnsongrass (*Sorghum halepense*) and large crabgrass (Ikley et al., 2015). Cn enters leaf tissues causing a foliar infection through wounds made by damage from hail, blowing sandy soil, farming machinery, and/or herbivores (Wysong et al., 1981; Rocheford et al., 1985; Agarkova et al., 2011). During heavy rain or hail, bacteria in infected debris splashes onto wounded leaves, allowing Cn to enter plant tissues. Disease symptoms first appear in the form of gray lesions with streaks of small water soaked spots (freckles), before turning necrotic. Bacterial exudates appear shiny after drying on the infected tissues (Langemeier et al., 2016). After colonizing the xylem vessels and spreading throughout the plant (Claflin, 1999), Cn may colonize the seeds and provide another source of inoculum (Schuster, 1975). Infection in absence of wounding can also occur via natural openings under increased humidity conditions (Mallowa et al., 2016).

Corn is a major cash crop for growers in the United States and Canada, and Goss's wilt and leaf blight disease is an economically damaging disease in most regions of North America (Mueller et al., 2016). In recent years, the disease has re-emerged and has spread throughout

* Corresponding author.
E-mail address: Fouad.Daayf@umanitoba.ca (F. Daayf).

https://doi.org/10.1016/j.cropro.2018.05.022
Received 14 March 2018; Received in revised form 23 May 2018; Accepted 27 May 2018

all major corn growing regions in the USA and Canada. In Canada, Goss's wilt disease has been identified in several provinces including Manitoba, Alberta, Ontario, and Québec (Zhu et al., 2000; Howard et al., 2015). This has prompted new research initiatives, but the lack of a consistent and reliable inoculation method has been a challenge in studying this disease. The methods used in other plant species to assess the pathogen's effects on the host included the injection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) into lower tomato stems with a gauge needle (Balaji et al., 2008), filling the whorl with large amounts of Cn, or Stewart's wilt inoculum (Pataky, 1985), cutting the leaf tip and inserting inoculum solution, and using pinprick-sponge soaked with Stewart's bacterial leaf blight inoculum (Chang et al., 1977; Suparyono and Pataky, 1989). Other inoculation methods previously used trimming devices with a spray attachment that helped produce a wound and introduced inoculum, but no validation data for disease assessment was presented other than percentage of infected plants (Cunfer et al., 1980).

Singh et al. (2016) studied leaf cut and dip, and machine inoculation methods on corn lines to assess their levels of tolerance to Goss's wilt. The machine inoculation method gave better separation of corn tolerance than the cut and dip methods. This method would possibly mimic leaf breakage, but the method for assessing the infected leaf area was not clear. Recently, Ahmad et al. (2015) reported a leaf inoculation method for corn, involving cutting across the leaf and applying a measured amount of inoculum. None of these studies attempted to validate the effectiveness or reproducibility of their selected inoculation method.

Developing robust high throughput inoculation methods and methods of disease assessment for field trials is necessary for breeders to evaluate the level of tolerance of corn lines against Goss's wilt and leaf blight disease. The methods developed in this study will help conduct projects aimed at studying interactions of corn with Cn under controlled conditions. This study may also provide breeders with a consistent method for screening levels of disease tolerance of corn parental lines under greenhouse conditions prior to designing breeding trials.

The objectives of this study were: (i) isolation and identification of Cn bacteria from samples collected from corn fields showing Goss's wilt symptoms in Manitoba, (ii) developing an easy-to-use, consistent, and reliable method for corn inoculation with Cn, and establishing a standardized disease severity scale using 2014 isolates, (iii) validation of the newly developed disease assessment methods using isolate collections from the 2015 and 2016 field seasons. and (iv) using the validated disease assessment methods under greenhouse conditions to compare isolate aggressiveness (The term aggressiveness in this study represents the level of damage caused by the Cn isolates) in subsequent field seasons.

2. Materials and methods

2.1. Sample collection

We collected corn leaf samples that showed typical Goss's wilt symptoms, including necrotic lesions with small water-soaked spots (Freckles) with wavy margins and sticky bacterial exudates that were shiny when dry, from 7, 24, and 24 corn fields in 2014, 2015, and 2016, respectively, in southern Manitoba (Fig. 1).

2.2. Detection and isolation procedures for Cn

2.2.1. Screening leaf tissues and Cn isolation

Leaves with visual symptoms were sampled using sterilized razor blades and screened with Agdia ImmunoStrip® (ISK44001 A&L Canada), advertised for detection of *Clavibacter michiganensis* (Cm). Potential positive tissues were surface sterilized using 10% commercial bleach (0.6% sodium hypochlorite), followed by rinsing three times with autoclaved reverse osmosis (RO) water in a laminar airflow hood and then macerated in autoclaved RO water in falcon tubes. The tubes were centrifuged at 4,000 g and the supernatant (100 µL) streaked onto both nutrient broth yeast extract (NBY), which contained; 8 g/L nutrient broth, 2 g/L yeast extract, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 5 g/L glucose and 1 mL of 1M $MgSO_4.7H_2O$, and on modified Cn selective CNS (*Corynebacterium nebraskense* selective media) plates (NBY supplied with 25 mg/L nalidixic acid, 32 mg/L polymyxin B sulfate, and 10 g/L LiCl) (Gross and Vidaver, 1979). The CNS media plates were incubated at room temperature (23–24 °C) for 7–10 days, and the apricot or orange color colonies were selected and re-plated on NBY for 3–4 days. Two to 3 colonies from each field were selected based on colony morphology. The selected colonies were subjected to Gram-stain procedure, (BD Gram stain kit Fisher Scientific Company) to confirm they were Gram-positive bacteria.

2.2.2. DNA extraction and PCR amplification

Genomic DNA was extracted from the isolates in pure culture using the MasterPure Gram-positive DNA purification kit (Epicentre Technologies, Illumina, Madison, Wisconsin, USA). PCR reaction was performed with a specific set of primers (PSA7-F, 5′ CCCTTTCCGTCG TCCTTTCG 3'; PSA7-R, 5′ TACTGAGATGTTTCACTTCCCC 3′), which gave a band size of 400 bp (Pastrik and Rainey, 1999). The PCR mixture was assayed using Dream Taq DNA polymerase (Fisher Scientific, Hampton, NH, USA) in 25-µL final volume with 10 ng gDNA template from each of the Cn isolates. In addition, positive and negative controls consisting of a previously identified Cn isolate, and *Bacillus subtilus*, respectively were assayed under the same conditions. The procedures were carried out according to the manufacturer's instructions. PCR amplification was run in the Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, California, USA) with an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 60 °C for 30s and extension at 72 °C for 60s, and a final extension at 72 °C for 10 min. The rate of the heating increase from 60 to 72 °C was 0.5 °C/s. The PCR product was run in 1% agarose gel supplied with 0.01% ethidium bromide. The amplified bands were visualized by Alpha Innotech gel documentation system (AlphaImager HP, Fisher Scientific, USA).

2.2.3. Pathogenicity assay of Cn isolates

All Cn isolates from southern Manitoba from the 2014, 2015 and 2016 seasons that tested positive for Cn by PCR were assessed for their pathogenicity (here, the ability to induce disease symptoms) on corn using the described leaf inoculation method in the following section. Three corn plants were inoculated and assayed for each tested Cn isolate. All isolates which generated typical Goss's wilt symptoms were considered pathogenic, numbered, and then maintained in glycerol stock at −80 °C for further studies.

2.3. Plant materials and growth conditions

Untreated seed from two corn hybrids, P7443 Pioneer (rated 4 on a 1–9 scale, 1 = susceptible, 9 = tolerant, Goss's wilt brochure, Pioneer Hi-Bred International, Inc. Johnston, IA, USA) and A4631G2 RIB Pride Seeds rated “VG” on a scale from Good to Excellent (2018 PRIDE Yield Guide Western Canada, Pride Seeds) were used in this study. All tested corn plants were grown under controlled conditions in a growth room with temperature and light period of 22/18 °C and 16/8 h light/darkness (Phillips high output T54), respectively. Corn plants were transplanted into 15 cm (1.89L) pots containing Sunshine® Mix #4 (Sungro Horticulture, Agawam, MA 01001–2907 USA) one week after seeding and inoculated 2–3 weeks later.

2.4. Leaf inoculation procedure

The leaf inoculation experiment was performed on three corn plants at the V4-V5 stage, where the 3rd, 4th, and 5th leaves of each plant